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Follistatin Is a Modulator of Gonadal Tumor Progression and the Activin-Induced Wasting Syndrome in Inhibin-Deficient Mice*

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ABSTRACT

Inhibins and activins are dimeric proteins belonging to the transforming growth factor-β superfamily. Follistatin is an activin-binding protein that antagonizes the function of activin via binding to its β-subunits. Previously, we demonstrated that mice deficient in inhibin develop ovarian and testicular sex cord-stromal tumors of granulosa and Sertoli cell origin, with 100% penetrance as early as 4 weeks of age. Overproduction of activins in the serum directly causes a cachexia-like wasting syndrome that results in lethality of these mice at an early stage after the onset of the tumors. In an independent set of studies, overexpression of mouse follistatin using the mouse metallothionein I promoter in transgenic mice led to gonadal defects and eventual infertility, primarily due to local effects of follistatin in these tissues. Activin has a positive growth effect on gonadal tumor cells in culture and directly causes the cancer cachexia-like syndrome in inhibin-deficient mice via interaction with activin receptor type IIA in livers and stomachs. We therefore hypothesized that an activin antagonist such as follistatin can act as a physiological modifier, either locally or via the serum, to block the activin-mediated cancer cachexia-like syndrome in inhibin-deficient mice and/or slow the progression of gonadal cancers in these mice. To test this hypothesis, we generated mice that are homozygous mutant for the inhibin α null allele (*i.e.* $inha^{m1}/inha^{m1}$) and carry the mouse metallothionein I follistatin (MT-FS) transgene. Our results show that gonadal tumors that are histologically similar in most, but not all, cases to the tumors in inhibin-deficient mice develop in these $inha^{m1}/inha^{m1}$, MT-FS^{+} mice. However, $inha^{m1}/inha^{m1}$, MT-FS^{+} mice exhibit a less severe wasting syndrome, lower serum activin levels, and a statistically significant prolonged survival in a number of cases compared with mice deficient in inhibin alone. Thus, follistatin can act as a modulator of tumor growth and the activin-induced cancer cachexia-like syndrome in inhibin-deficient mice. (*Endocrinology* **141:** 2319–2327, 2000)

INHIBINS and activins were initially identified as gonadal peptides that inhibit or stimulate, respectively, pituitary FSH production (1). These peptides have since been shown to play diverse roles in development, growth and differentiation, and various physiological processes (1–6). Inhibins are α:β heterodimers (α:βA or α:βB), whereas activins are homodimers (βA:βA; βB:βB) and heterodimers (βA:βB) that share a common β-subunit with inhibin. Inhibins are expressed in multiple tissues, with the highest levels of expression in the Sertoli cells of the testis, the granulosa cells of the ovary, and the pituitary (2).

To study the roles of inhibins in mammalian reproduction and development, we previously generated inhibin knockout mice (7). These inhibin-deficient mice develop ovarian and testicular sex cord-stromal (Sertoli cell and/or granulosa cell) tumors with 100% penetrance, which were evident as early as 4 weeks of age in both sexes. Inhibin-deficient male mice developed testicular tumors that typically were bilateral, hemorrhagic, and multifocal (7), often resembling juvenile granulosa cell tumors found in young girls. Consistent with these histological findings, serum from these male mice demonstrated elevated estradiol levels (3). Female inhibin-deficient mice developed multifocal, hemorrhagic ovarian tumors that were often mixed granulosa and Sertoli cell tumors (7) and also showed elevated estradiol levels in most cases (3). As the tumors progressed, extensive destruction of the normal gonadal architecture by the focally invasive tumor occurred, leading to significant hemorrhage (7).

Gonadal tumor development in inhibin-deficient mice was accompanied by a severe progressive cancer cachexia-like wasting syndrome characterized by weight loss, lethargy, kyphoscoliosis, and a sunken eye appearance. The pathological findings of this wasting syndrome included anemia, hepatocellular necrosis around the central vein of the liver, and atrophy in the glandular stomach with a block in differentiation of multiple gastric lineages (8, 9). In addition to estradiol, serum levels of activin A and B and FSH were elevated in the inhibin-deficient mice after the tumors developed (3, 7–9). The above-mentioned effects of activins on the liver and stomach are due to direct effects of activins signaling through activin receptor type IIA (9, 10). Subsequently, using ovary transplantation techniques, we proved that absence of inhibin, rather than elevated levels of activin, caused the tumor development in these inhibin-deficient mice, thus defining inhibin as a tumor suppressor in the

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gonads (3). Inhibin also acts as a tumor suppressor in the adrenal glands; nearly 100% of inhibin-deficient mice developed adrenal cortical tumors after castration (7, 8). Although inhibin secretion from a contralateral ovary can suppress tumor development in an inhibin knockout ovary (3), and absence of activin receptor type IIA does not prevent ovarian or testicular tumor development (10), activin secretion from the tumor cells can stimulate the growth of these inhibin-deficient gonadal tumor cells in culture via an autocrine mechanism (11). In addition, follistatin added to the culture medium slows the growth rate of the tumor cells (11).

Follistatin is structurally unrelated to activins or inhibins. Follistatin binds to the β-subunits of activin to prevent the interaction of activin with its type II receptors (12, 13). *In vivo*, this leads to inhibition of FSH synthesis and secretion (14). Follistatin may be important in regulating other factors besides activin, since it binds to other transforming growth factor-β superfamily members such as bone morphogenic protein-4 (BMP-4), BMP-7, and inhibin (15–17). The affinities of follistatin for BMP-4 and BMP-7 are not known, but are presumed to be less than the affinity for activin (15, 16). Although follistatin is expressed in multiple tissues in mammalian development (2, 3, 12, 18–21), the ovarian granulosa cells are the major site of its production (18, 21). We previously showed that follistatin knockout mice demonstrate widespread dermatopathological and musculoskeletal defects and growth delay (22). These defects are more diverse than those seen in activin receptor type IIA (ActRIIA)-, ActRIIB-, or activin-deficient mice (23–25). Follistatin-deficient mice die within hours of birth, preventing any further functional studies of follistatin in reproductive development and physiology. We subsequently generated a transgenic mouse model in which mouse follistatin (FS) was overexpressed using a mouse metallothionein (MT) I promoter (26). The presence of the follistatin transgene (MT-FS$^+$) did not affect viability, but the transgenic mice had defects in fertility (26). MT-FS$^+$ male mice exhibited Leydig cell hyperplasia, arrested spermatogenesis, and seminiferous tubule degeneration, which contributed to the infertility in these mice. MT-FS$^+$ female mice had small ovaries, with blocks at different stages of folliculogenesis, and thin uteri and were infertile. Dermatological defects were present in both follistatin knockout and MT-FS$^+$ transgenic mice, but there was no evidence of cancer.

Because of the contribution of activin to the liver and stomach phenotypes in the inhibin-deficient mice and its stimulatory effect on tumor cell growth in culture, we hypothesized that overexpression of follistatin in the inhibin α knockout background would antagonize activin's actions and possibly prevent the cancer cachexia-like syndrome and/or alter the tumor growth rate. To test this, we generated mice that were homozygous mutant for inhibin α and carried the MT-FS transgene (*inha*^{m1}/*inha*^{m1}, MT-FS$^+$). Here, we demonstrate that overexpression of follistatin slows the cachexia-like wasting syndrome and the progression of tumor development in a number of inhibin-deficient mice.

Materials and Methods

Generation of inha^{m1}/inha^{m1}, MT-FS$^+$ mice

Generation of inhibin heterozygous (*inha*^{m1}/+) and homozygous (*inha*^{m1}/*inha*^{m1}) mutant mice and mice carrying multiple copies of the MT-FS follistatin transgene (MT-FS$^+$) were previously described (7, 26). Heterozygous inhibin α and MT-FS transgenic (line 5) male and female mice were crossed to generate offspring that were homozygous mutant for inhibin α and carried the MT-FS transgene (*inha*^{m1}/*inha*^{m1}, MT-FS$^+$). These mice were obtained at the expected Mendelian ratio of 3:16.

Southern blot analysis

To determine the inhibin α genotype of offspring, Southern blot analyses were performed on tail DNA samples using ^{32}P-labeled probes as previously described (7). Detection of the MT-FS transgene using a 973-bp simian virus 40 3′-untranslated region/polyadenylated fragment as the probe was described previously (26).

Morphological and histological analysis

Mice from each genotype were weighed once a week beginning at 4 weeks. Mouse ovaries, testes, stomachs, and livers were fixed in either 10% buffered formalin or Bouin's fixative overnight. Bouin's-fixed tissues were then transferred to 70% ethanol saturated with lithium carbonate and washed extensively. Tissue sections were dehydrated and paraffin wax embedded, and 4-μm sections were cut and stained with hematoxylin and eosin or periodic acid-Schiff. Gross and histological analyses of the tissues were performed on at least six male and six female mice.

Serum analysis

Mice were anesthetized using Metofane (Schering Plough Animal Health Corp., Union, NJ), and serum was collected using either cardiac puncture or capillary eye bleed in Microtainer tubes (Becton Dickinson and Co., Franklin Lakes, NJ). Serum activin A levels were determined using an activin A enzyme-linked immunosorbent assay kit from Serotec (Kidlington, Oxford, UK). The standard used was bovine follicular fluid. This assay measures total activin A levels in serum (both free and bound). The sensitivity of this assay is 3.8 pg/ml. The Serotec assay has been validated for mouse serum (Dr. Teresa K. Woodruff, personal communication). FSH levels were determined using a [^{125}I]hormone labeling method as previously described (27). Serum samples used for FSH analysis were obtained from the same group of mice as that previously used for activin A serum analysis. FSH values are represented as the mean ± SEM. The sensitivity of the FSH assay is 10 ng/ml, and the NIDDK standard used was rat FSH I-9.

Statistical analysis

A confidence interval test was used to determine the significance of the survival rates. One-way ANOVA was used to determine the statistical significance of serum samples. Microsoft Corp. Excel (Redmond, WA) was used for both of these analyses. $P < 0.05$ was considered significant.

Results

The inha^{m1}/inha^{m1}, MT-FS$^+$ mice develop a less severe wasting syndrome and survive longer

By approximately 6–7 weeks of age, inhibin-deficient male and female mice begin to lose weight, indicative of a cancer cachexia-like wasting syndrome secondary to the production of activins by ovarian and testicular tumors (8) (Fig. 1). Before this time, the weight gain of these mice is indistinguishable from that of their wild-type littermate controls as are the serum activin A and B levels (8). In the present study we show that in contrast to inhibin-deficient mice or transgenic mice carrying only the MT-FS transgene, growth curves of male (Fig. 1A) and female (Fig. 1B) mice overexpressing the follistatin transgene and lacking inhibin (*inha*^{m1}/*inha*^{m1}, MT-FS$^+$) were intermediate between those of the cachectic-prone *inha*^{m1}/*inha*^{m1} mice and the healthy MT-FS$^+$ mice. Although the *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice did exhibit some weight loss,

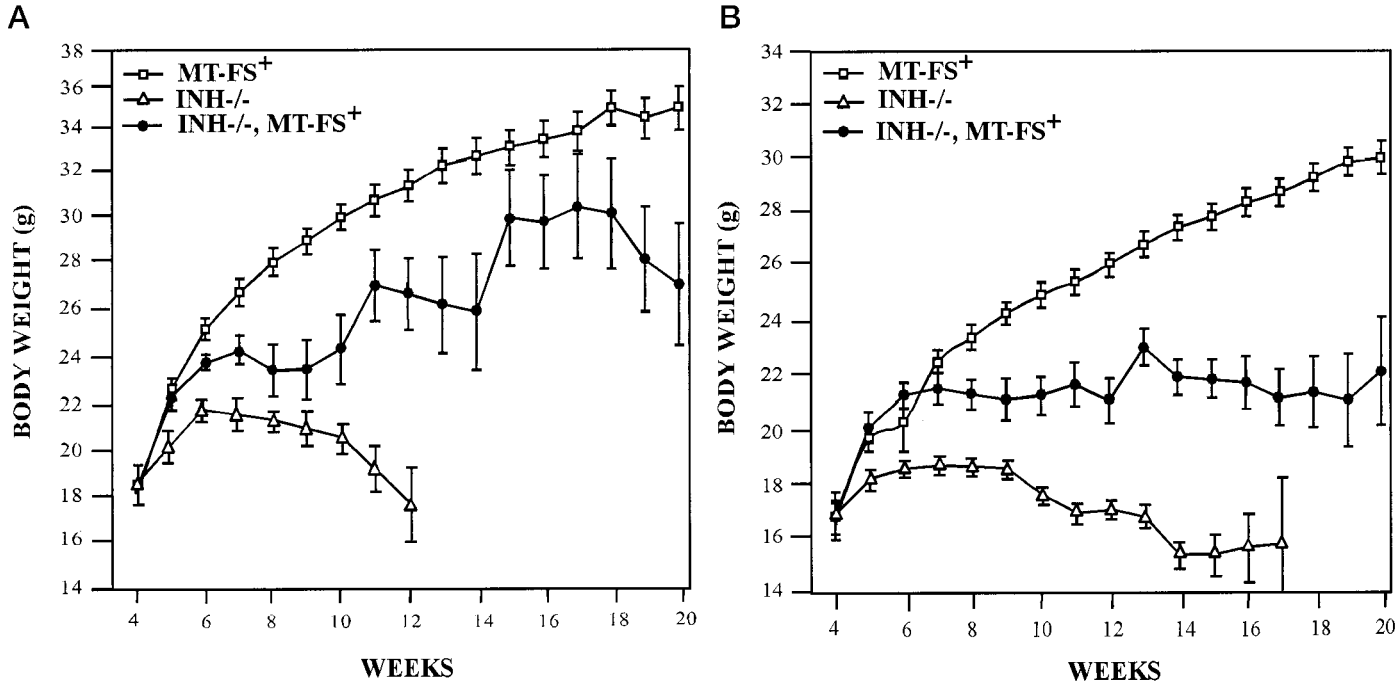

FIG. 1. Weekly body weights for male (A) and female (B) MT-FS$^+$, INH$^{-/-}$ (*inha*^{m1}/*inha*^{m1}), and INH$^{-/-}$, MT-FS$^+$ (*inha*^{m1}/*inha*^{m1}, MT-FS$^+$) mice. Values at each point represent the mean ± SEM. The number of mice used were: MT-FS$^+$, 19 males and 20 females; INH$^{-/-}$, 38 males and 52 females; and INH$^{-/-}$, MT-FS$^+$, 20 males and 24 females.

this cachexia was less dramatic, and there were minimal other symptoms [*e.g.* less of a hunchback appearance (kyphoscoliosis)] during the 20-week period compared to mice lacking only inhibin. One hundred percent of MT-FS$^+$ control male and female mice were alive after 20 weeks (Fig. 2, A and B). This correlates with normal weight gain in the MT-FS$^+$ mice over this time period (Fig. 1, A and B). At 13 weeks of age, 97% of the *inha*^{m1}/*inha*^{m1} males were dead, in contrast to 40% of the *inha*^{m1}/*inha*^{m1} MT-FS$^+$ mice (Fig. 2A). At 17 weeks of age, 95% of the *inha*^{m1}/*inha*^{m1} female mice were dead, whereas 38% of the *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ female mice were still alive. Even at 20 weeks, there was a statistically significant number of the *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ female mice that were still alive (21%, 5 of 24) compared with *inha*^{m1}/*inha*^{m1} female mice (2%, 1 of 52; confidence interval, 0.189 ± 0.085; $P < 0.001$). Similarly, after 20 weeks of age, a statistically significant number of *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ male mice were alive (20%, 4 of 20) compared with *inha*^{m1}/*inha*^{m1} male mice (0%, 0 of 38; confidence interval, 0.2 ± 0.089; $P < 0.002$).

Over an 8-yr period of breeding to generate *inha*^{m1}/*inha*^{m1} mice, we observed hundreds of inhibin-deficient mice, including our published reports (3, 7, 8, 10, 27) and unpublished data. As mentioned above, more than 95% of the inhibin-deficient male and female mice will die from the cachexia-like syndrome by 12 and 17 weeks, respectively, and this frequency has not changed over time. Until now, only genetic manipulation such as deletion of activin receptor type IIA (10) or absence of one or both gonadotropins (27, 28) alters the survival rate past this point (see *Discussion*). In addition, of these several hundred inhibin knockout mice that have been generated, the oldest male has lived to 17 weeks, and only a single female has survived past 20 weeks (dying before 22 weeks; see Fig. 2A). However, as mentioned above, nine *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice were still alive at 20 weeks of age. Furthermore, we observed six *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice that survived between 6 and 8 months of age.

The inha^{m1}/inha^{m1}, MT-FS$^+$ mice develop ovarian and testicular tumors histologically similar to those in inha^{m1}/inha^{m1} mice

As might be expected from the weight loss and survival curves (Figs. 1 and 2), *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice do develop gonadal tumors. Tumor formation in the *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ male and female mice was first evident in some mice at 7 weeks of age, and by 12 weeks of age, tumors were grossly evident in a number of these mice (Fig. 3, A and B). Histological analysis revealed that *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ male and female mice developed multifocal undifferentiated Sertoli and/or granulosa cell tumorigenic lesions (Fig. 3, C and D). As mentioned above, our original reports of mice lacking only inhibin (3, 7, 10, 27) demonstrated that the testicular tumors typically resembled human juvenile granulosa cell tumors of young females and produced high levels of estradiol. Interestingly, the tumors in the *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ male mice also had components that resembled Sertoli cell tumors, which were never seen in mice lacking only inhibin (3, 7, 10, 27) (Matzuk and colleagues) and which were more typical of human and mouse mixed granulosa/Sertoli cell tumors (see Fig. 3F). Female mice lacking inhibin and carrying the MT-FS transgene also predominantly showed mixed granulosa/Sertoli cell tumors with distinct areas of mitotically active granulosa cells (Fig. 3D). The extent of

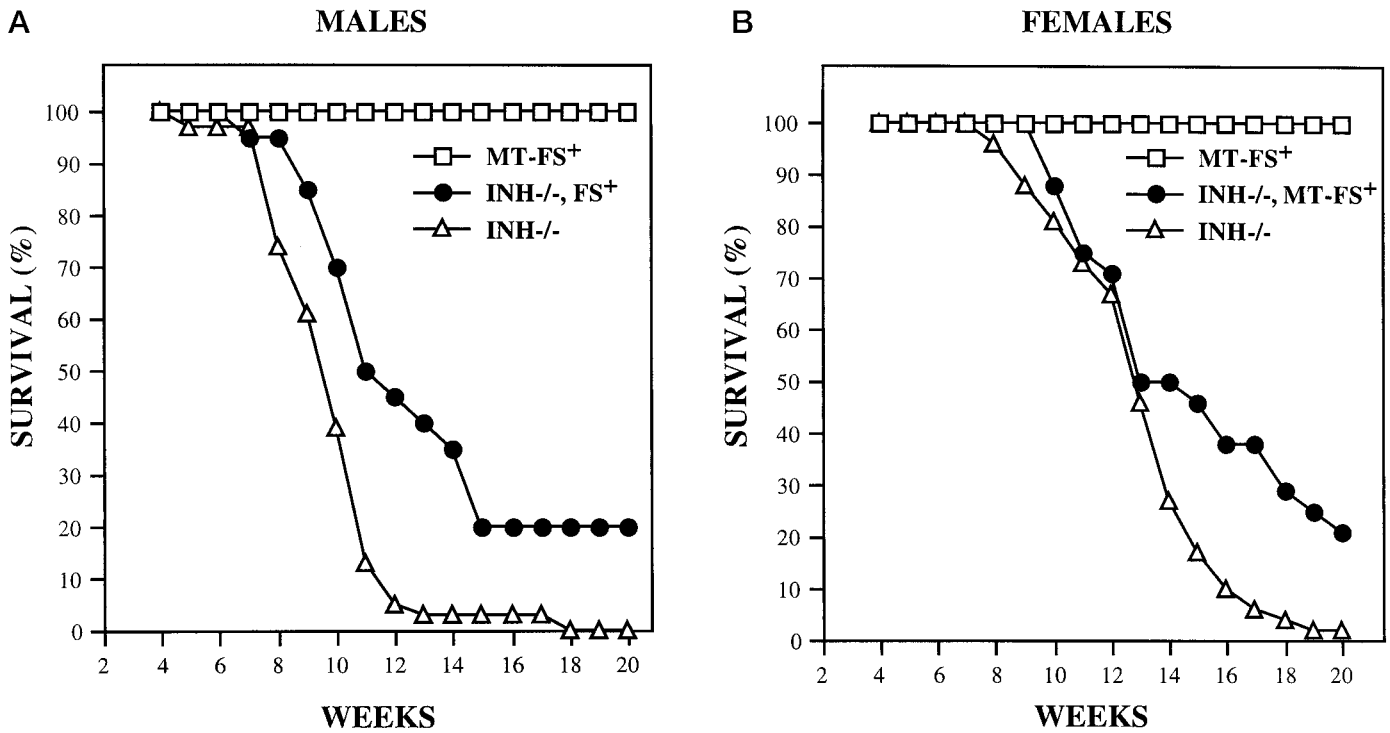

FIG. 2. Survival curves for male (A) and female (B) MT-FS, INH$^{-/-}$ (*inha*^{m1}/*inha*^{m1}), and INH$^{-/-}$, MT-FS^{+} (*inha*^{m1}/*inha*^{m1}, MT-FS^{+}) mice. Mice were counted once per week. The number of mice used were: MT-FS^{+}, 19 males and 20 females; INH$^{-/-}$, 38 males and 52 females; and INH$^{-/-}$, MT-FS^{+}, 20 males and 24 females.

hemorrhage within the tumor tissue of both male and female *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mice did not correlate with a specific tumor cell type. We noted, however, that in contrast to inhibin-deficient mice, only sparse areas of focal hemorrhage were present in the ovaries of *inha*^{m1}/*inha*^{m1}, MT-FS^{+} female mice. Thus, the presence of the MT-FS transgene in the inhibin-deficient background appears to somehow alter the tumorigenic process in both male mice (*i.e.* increased Sertoli cell tumor component) and female mice (*i.e.* less hemorrhage noted; see *Discussion*).

As mentioned above, we observed, two *inha*^{m1}/*inha*^{m1}, MT-FS^{+} male mice surviving to 6–8 months of age. In the 6-month-old male, gross analysis showed the testes to be enlarged and bilaterally hemorrhagic. Similarly, in the 8-month-old male, both testes were grossly enlarged, with marked hemorrhage. A section from one of these testicular tumors demonstrated that it was histologically similar (Fig. 3E) to the granulosa cell tumor component seen in some *inha*^{m1}/*inha*^{m1}, MT-FS^{+} females (Fig. 3D) and more typical of testicular tumors from male mice lacking only inhibin (3, 7, 10, 27). The bilateral tumors from one of the 8-month-old *inha*^{m1}/*inha*^{m1}, MT-FS^{+} females were grossly larger than the tumors taken from an *inha*^{m1}/*inha*^{m1}, MT-FS^{+} 12-week-old mouse (Fig. 3B). Histologically, these tumors were mixed granulosa/Sertoli cell tumors. However, the Sertoli cell component of these tumors (Fig. 3F), which was histologically similar to the testis tumors (Fig. 3C), predominated and was a major portion of the tumor. Interestingly, the gross (Fig. 3A) and histological (Fig. 3, E and F, and data not shown) analysis of the two tumors from these older female and male *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mice demonstrated only sparse hemorrhage, suggesting that the tumors were less aggressive locally. In addition, all of the 6- to 8-month-old male and female mice failed to develop any cachexia-like symptoms (see next section).

The inha^{m1}/inha^{m1}, MT-FS^{+} mice have reduced serum activin A levels compared with inha^{m1}/inha^{m1} mice

Gonadal tumors from inhibin-deficient male and female mice secrete activin A and B into the serum at high levels (8–10). These circulating activins cause a cachexia-like wasting syndrome. To address whether the decreased weight loss and prolonged survival of the *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mice could be due to reduced serum activin levels, we performed activin A ELISAs. We used serum from 14- to 25-week-old *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mice and 13- to 18-week-old *inha*^{m1}/*inha*^{m1} mice. These time points correlated with periods of decreased survival and weight loss. The highest levels of serum activin A were detected in *inha*^{m1}/*inha*^{m1} male and female mice (Fig. 4, A and B, respectively). For males and females, serum activin A levels in *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mice were statistically lower than those in *inha*^{m1}/*inha*^{m1} mice, although they continued to be elevated compared with levels in control MT-FS^{+} mice (Fig. 4; $P < 0.05$). Serum activin A levels in MT-FS^{+} male and female control mice were essentially undetectable (Fig. 4, A and B; males, 0.16 ± 0.01 ng/ml; females 0.25 ± 0.02 ng/ml). Interestingly, when the serum activin A levels were analyzed in the 8-month-old female and the 6-month-old male *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mice, the levels of activin A were extremely low (5.23 ng/ml and undetectable, respectively). Thus, despite the presence of gross tumors in these two mice, activin levels were not dra-

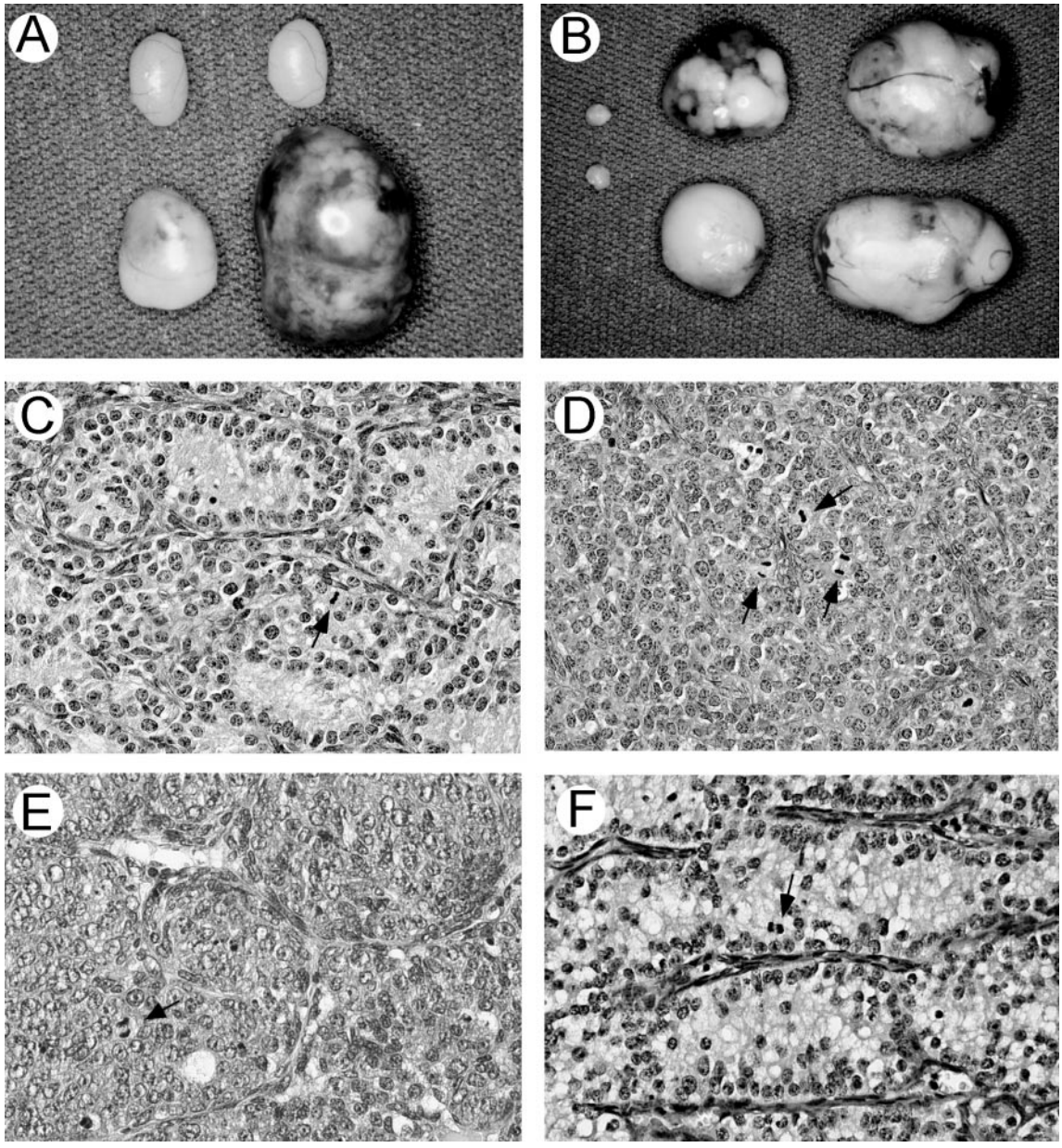

FIG. 3. Morphological and histological analysis of testes and ovaries from control and mutant mice. A, Morphological analysis comparing testes from a MT-FS$^+$ control mouse (*upper part of panel*) and a 12-week-old *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mouse (*lower part of panel*). As shown, testicular tumors were grossly enlarged and hemorrhagic compared with the wild-type control testes. B, Gross analysis of ovaries from a wild-type mouse (*left part of panel*), 12-week-old (*middle*) and 8-month-old (*far right*) *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice. The ovaries from the 12-week-old and 8-month-old *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice were grossly enlarged compared with the control. C, Histological examination of a testicular tumor from a 12-week-old *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ male at higher magnification demonstrates a mitotically active undifferentiated sex cord-stromal tumor in a Sertoli cell tubular arrangement (compare with F). Tumors of this type were never seen in mice lacking only inhibin. D, Shown at high power magnification is a section of mitotically active granulosa cell tumor taken from the 12-week-old *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ female in B above. E, Section of a testicular tumor from an 8-month-old male *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mouse at high power magnification. The histology is more consistent with a granulosa cell tumor (compare with D). F, Section of the ovarian tumor at high power magnification taken from the 8-month-old *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ female in B showed extensively disorganized tissue consisting of a primarily undifferentiated Sertoli cell tumor. The *arrows* in C, D, E, and F indicate obvious mitotic figures.

matically elevated and may have caused the prolonged survival of these mice (see *Discussion*).

Interestingly, analysis of FSH serum levels in these *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice revealed opposite trends in males and females *vs.* controls. The *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ male mice (n = 5) had a mean FSH level of 39.6 ± 8.2 ng/ml (*vs.* control MT-FS$^+$ male level of 68.7 ± 9.3 ng/ml; n = 9), whereas the FSH level in *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ female mice (n = 9) was 73.6 ± 13.84 ng/ml (*vs.* control MT-FS$^+$ female level of 39.1 ± 8.4 ng/ml; n = 10). Thus, there appears to be paradoxical effects of the presence of the MT-FS transgene in male and female inhibin-deficient mice (see *Discussion*).

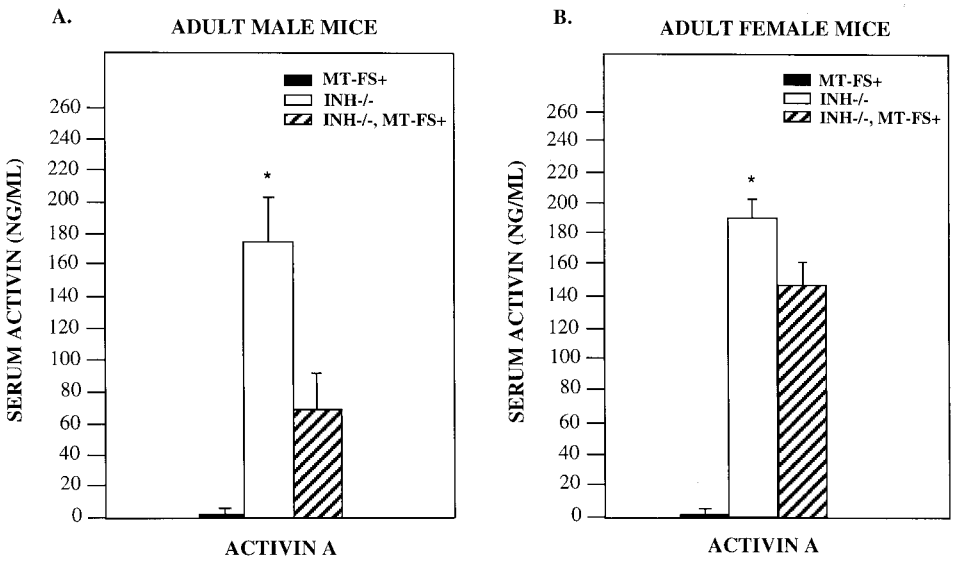

FIG. 4. Serum activin A levels for adult male (A) and female (B) mice. Activin A levels for MT-FS$^+$ mice (control), INH$^{-/-}$ (*inha*^{m1}/*inha*^{m1}), and INH$^{-/-}$, MT-FS$^+$ (*inha*^{m1}/*inha*^{m1}, MT-FS$^+$) mice are represented. Mice used were between the ages of 14 and 25 weeks of age. Values at each point are the mean ± SEM. Mice used in this analysis were MT-FS$^+$ (9 males and 10 females), INH$^{-/-}$ (16 males and 7 females), INH$^{-/-}$ MT-FS$^+$ (8 males and 6 females). The *asterisks* represent a statistically significant difference between serum levels of INH$^{-/-}$ male and female mice and those of MT-FS$^+$ and INH$^{-/-}$ MT-FS$^+$ male and female mice, respectively.

The inha^{m1}/inha^{m1}, MT-FS$^+$ mice have less severe stomach and liver pathology compared with inha^{m1}/inha^{m1} mice

The cachexia-like wasting syndrome in the *inha*^{m1}/*inha*^{m1} mice is due to increased activin secretion from the gonadal tumors (8, 10). This syndrome results in increased signaling through ActRIIA, which results in liver and stomach pathology in these mice (8–10). To confirm that the wasting syndrome in many of the *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice was due to a similar pathological process, we examined these tissues histologically. Indeed, compared with the essentially normal glandular stomach of *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice at 7 weeks (Fig. 5A), parietal cells were mildly depleted in the glandular stomach of a cachectic *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mouse at 12 weeks (Fig. 5B). The pathology in these mice was milder than the complete and marked atrophy seen in the stomachs of *inha*^{m1}/*inha*^{m1} mice (*i.e.* nearly complete disappearance of parietal cells and other lineages, as determined by light and immunohistochemical analysis) (8, 9). The stomachs of the 8-month-old female and 6-month-old male were also analyzed. In the 8-month-old female (Fig. 5C) and 6-month-old male (data not shown) *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice, stomach histology appeared completely normal with no obvious depletion of parietal cells, consistent with the low serum activin A levels.

Livers of *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice at 7 and 12 weeks of age were more abnormal, showing slight, diffuse inflammation around the central vein but no dramatic necrosis (Fig. 5, D and E). However, the livers of the 8-month-old female (Fig. 5F) and 6-month-old male (data not shown) *inha*^{m1}/*inha*^{m1} MT-FS$^+$ mice failed to show any signs of hepatocellular necrosis or inflammation. Thus, although tumors occurred in the older male and female *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice, these mice lived longer because of the low activin levels and the lack of resultant liver and stomach pathology.

Discussion

The control of growth and differentiation in the gonads is a complex process involving a number of endocrine, paracrine, and autocrine factors, including inhibins, activins, and follistatin (29, 30). Transgenic mice overexpressing follistatin (MT-FS$^+$ mice) have reproductive defects, including infertility, due to a direct effect of follistatin in the ovaries and testes (26). Mice lacking inhibin develop sex cord-stromal (granulosa/Sertoli cell) tumors at 100% penetrance and an accompanying cancer cachexia-like syndrome as a result of tumor-produced activins and signaling through the activin receptor type II in liver and glandular stomach (3, 7–10). Activins also stimulate the growth rate of these tumor cells in culture via an autocrine mechanism, and follistatin blocks this effect (11). We therefore hypothesized that overexpression of follistatin in the background of inhibin deficiency could modulate the tumor growth rate and/or the cancer cachexia-like syndrome via its ability to block activin effects.

In the present study we show that overexpressing follistatin in the absence of inhibin did not prevent initial gonadal tumor growth in mice. Sex cord-stromal tumors continued to develop in 100% of the *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ male and female mice. However, we show that follistatin in the absence of inhibin is an important modulator of gonadal tumor progression and/or cachexia in the inhibin-deficient mice. The presence of the MT-FS transgene resulted in prolonged survival despite intermediate weight loss and a significant tumor burden. A higher proportion (~20%) of both male and female *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice survived longer than 20 weeks of age compared with inhibin-deficient male mice, which never survived past 17 weeks, and inhibin-deficient females, of which only one rare female survived past 20 weeks of age. In addition, a total of six *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice lived to 6–8 months of age, further confirming the important role of follistatin as a modulator in these mice.

How is follistatin exerting its effects to increase the survival of these inhibin-deficient mice? It appears that follistatin is likely working via several mechanisms in these mice. First, at least in the case of the male *inha*^{m1}/*inha*^{m1}, MT-FS$^+$ mice, there appears to be altered tumor differentiation. We observed sex cord-stromal tumors with an increased Sertoli cell tumor component in these transgene-carrying male mice, a finding that was never seen in males lacking only inhibin

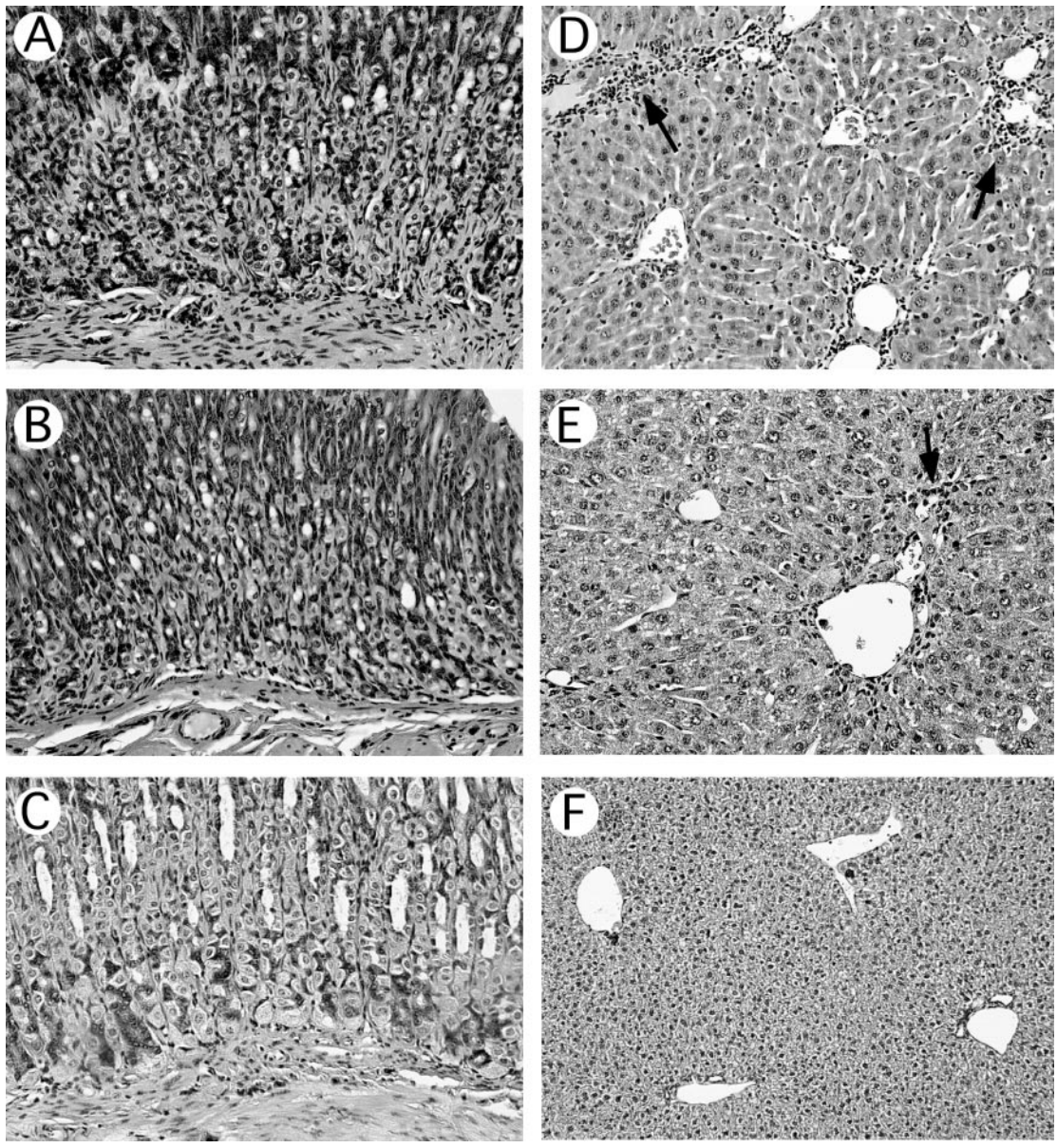

FIG. 5. Histological analysis of stomachs and livers of *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mice from 7-week-male (A and D), 12-week-old female (B and E), and 8-month-old female (C and F) *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mice viewed at the same magnification. In A, note the abundance of parietal cells in the glandular stomach of the 7-week-old mouse. In contrast, there is mild atrophy, and there was only mild depletion of parietal cells in the same region at 12 weeks in the *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mouse (B). No dramatic depletion of parietal cells was seen in the 8-month-old female *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mouse (C). Note the relatively normal appearance of the liver, showing only minimal inflammation at 7 and 12 weeks (D and E). In contrast, no destruction of liver architecture or inflammation was seen in an 8-month-old *inha*^{m1}/*inha*^{m1}, MT-FS^{+} female mouse (F). The *large arrows* in D and E indicate areas of inflammation.

(7) (Matzuk and colleagues). Second, activin levels from both the male and female *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mice were reduced compared with those in *inha*^{m1}/*inha*^{m1} mice, leading to less dramatic wasting effects (*i.e.* reduced stomach pathology) in these mice. This could be due to differences in the secretion of activin from the tumors (*e.g.* because of tumor differentiation), because local follistatin in the tumor is preventing the release of activin into the circulation (*e.g.* increased surface-bound follistatin binding the activin), or because follistatin-bound activin is cleared more rapidly from the circulation. This mechanism is likely the reason for the prolonged survival of the 6- to 8-month-old *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mice, which have very low levels of activin in the serum. Third, although we were unable to previously detect free follistatin in the circulation of MT-FS transgenic mice (26), this does not rule out the possibility that much of the activin in the circulation of *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mice is bound to follistatin and that this is reducing the overall positive effects of the circulating activins. This is possibly true for several reasons, including the lower FSH levels in these mice [compared with our previously published findings for inhibin-deficient mice (7) (see below)] and the mild effects in the stomachs of the transgene carriers [in contrast to the nearly complete atrophy in mice lacking only inhibin,

as observed at the histological and immunohistochemical levels (8, 9)]. We believe that all three of these mechanisms are having an effect in our mice, with some mechanisms being more prominent than others.

Why doesn't the presence of MT-FS transgene have the same effect in all inhibin-deficient mice? All mice lacking inhibin do not die at the same age (3, 7, 8). This is due to genetic differences in the mice, the timing of the oncogenic "hits," and differences in aggressiveness of the various tumors. Likewise and similar to other transgenic studies, all transgenes are not expressed the same in different mice even within a line. This difference in expressivity between the transgenic mice is one possible reason for the early death of some mice relative to others.

Our laboratory has generated several compound mutants to study gonadal tumor growth and differentiation. We previously generated double homozygous mutant mice deficient in both inhibin αand ActRIIA (10). Gonadal sex cord-stromal tumors developed in these compound homozygotes; however, the mice suffered no unusual weight loss, and the livers and stomachs were histologically normal (9, 10). These results demonstrated that the wasting syndrome in the inhibin-deficient mice was due to increased levels of activin signaling through the ActRIIA in hepatocytes and the glandular stomach. Several lines of evidence have also shown an important role of gonadotropins in regulating steroidogenesis and gonadal tumor development. Mice that lack both inhibin α and GnRH and have suppressed LH and FSH levels (27) do not develop gonadal or adrenal tumors or a wasting syndrome and can survive for more than 1 yr. These studies identified FSH and LH as modifying factors of tumor development in these mice. In confirmation of these studies, double mutant mice lacking both inhibin and FSH develop sex cord-stromal tumors, yet 70% of the males are still alive by 1 yr of age and have minimal cachexia, and double mutant females live twice as long as female mice deficient in only inhibin α (28). In these studies activin secretion from these tumors in the absence of FSH was dramatically decreased, suggesting that FSH acts in part as a modifier to reduce the cachexia-like symptoms. In the present studies both activin and FSH levels in the male *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mice are lower than the levels in the female mice, and as might be anticipated, levels of FSH are lower in these same males relative to those in females. The lower FSH levels in the *inha*^{m1}/*inha*^{m1}, MT-FS^{+} male mice, compared with those in female mice of the same genotype, could also be acting as an additional modifier of gonadal tumor development. Thus, gonadal tumor development and the cancer cachexia-like syndrome involve an extremely complex network of circulating and local factors, with inhibins, activins, follistatin, and gonadotropins playing key roles in these processes.

In human patients with advanced stage tumors and hematological malignancies, free follistatin levels were elevated (29). S1-Nuclease assays showed follistatin messenger RNA to be expressed in the testes, ovary, kidney, cerebral cortex, pituitary, adrenal, pancreas, heart, uterus, lung, and skeletal muscle of the rat (21, 30). Ovarian neoplasms account for only 4% of all cancers among women (31). However, the lack of early symptoms accounts for a 5-yr survival rate of only 46% for all stages and the highest mortality rate of cancers of the female reproductive system (31). Testicular cancers represent only 1% of all tumors in males, but is the most common malignancy in males between the ages of 15 and 34 yr (31, 32). Our present findings demonstrate a modulating role for follistatin in mouse gonadal tumorigenesis. Other hormonal factors may also influence initial tumor development and/or survival. For example, it will be interesting to determine whether signaling through estrogen receptors and/or LH receptor will influence gonadal tumor development and progression in inhibin mutant mice. The *inha*^{m1}/*inha*^{m1}, MT-FS^{+} mice may be an important *in vivo* model for furthering our understanding of the roles of follistatin in gonadal tumor development and the study and testing of markers for granulosa cell tumors.

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